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(54) Title: DNA SEQUENCES ENCODING A LYCOPENE CYCLASE, ANTISENSE SEQUENCES DERIVED THEREFROM AND THEIR USE FOR THE MODIFICATION OF CAROTENOIDS LEVELS IN PLANTS

(57) Abstract

The invention relates to DNA constructs comprising a DNA sequence homologous to some or all of a sequence encoding a lycopene cyclase, and to their use for modifying carotenoids levels in plants.

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DNA SEQUENCES ENCODING A LYCOPENE CYCLASE, ANTISENSE SEQUENCES DERIVED THEREFROM AND THEIR USE FOR THE MODIFICATION OF CAROTENOIDS LEVELS IN PLANTS

The invention relates to DNA constructs containing DNA sequences encoding a lycopene cyclase or containing antisense sequences of said DNA sequences, and their use for the modification of carotenoids levels in plants.

The invention also relates to processes for modifying the production of carotenoids in plants, and to plants or fragments thereof, or seeds transformed with said DNA constructs.

Plants and various photosynthetic or non-photosynthetic microorganisms synthesize a great number of different carotenoids (for a review see Spurgeon and Porter, 1980; Goodwin, 1980). These C40 compounds are formed from isoprene units and have been desaturated to produce a chromophore with conjugated double bonds. Carotenoids are well known as being essential components of the photosynthetic apparatus where they play important roles as light-harvesting pigments, as protectants against photooxidation as well as the assembly of these complexes.

In plants and cyanobacteria, phytoene (the precursor of all carotenoids) is converted to lycopene via four desaturation reactions catalyzed by two dehydrogenases (for a review see Sandmann, 1994). Lycopene is considered to be the normal precursor of cyclic carotenoids. Two types of cyclohexenyl rings are found in plant carotenoids: β- ring or ε- rings. In β-carotene and its derivatives, a β-ring is present at each end of the molecule, whereas α-carotene and its derivatives possess a β-ring at one end and an ε-ring at the other.

β-carotene is an important component in the reaction centers and antenna of the photosynthetic apparatus. It is also a substrate for the biosynthesis of the other important carotenoids, such as the xanthophylls zeaxanthin, antheraxanthin, violaxanthin, and neoxanthin. β-carotene via the above-mentioned xanthophylls is also a precursor of the phytohormone abscisic acid (Rock and Zeewart, 1991). In addition, β-carotene is the most important precursor of vitamin A in human food and animal feed (Olsen, 1989). On the other hand, lutein, an α-carotene derivative, is an abundant carotenoid in the photosynthetic apparatus of plant cells. The mechanism by which plant cells channel linear carotenoids in one or the other class of cyclic carotenoids is not well understood.

In some plants, non-photosynthetic cells are able to accumulate large amounts of carotenoids in specialized type of plastids called chromoplats. These carotenoids serve as visual attractants of animals facilitating pollination

or seed dispersal. A great diversity exists in chromoplast carotenoids which can be either predominantly of the linear type (e.g. lycopene in tomato fruits) or of the cyclic type (for a review see Goodwin, 1980). The latter are usually oxidized derivatives of either α-carotene or β-carotene. Many species-specific chromoplast carotenoids have been described, such as the ketocarotenoids capsanthin and capsorubin in *Capsicum annuum* fruits. The latter carotenoids contain one or two cyclopentane end groups (κ-ring) which result from a rearrangement of the epoxidized β-cycle(s) of antheraxanthin and violaxanthin respectively. Therefore, synthesis of these various carotenoids must be under tight control in these non-photosynthetic cells.

In order to study the mechanisms involved in the overaccumulation of carotenoids in chromoplasts, a number of relevant enzymatic activities have been characterized in *C. annuum*. More specifically, a lycopene cyclase, which has been found to operate in chromoplasts membranes (Camara et al., 1982) has been solubilized in an active form (Camara and Dogho, 1986). In a second step, various cDNAs have been cloned from this organism and characterized (Hugueney et al., 1992; Kuntz et al., 1992; Römer et al., 1993; Bouvier et al., 1994).

The invention relates to the use of recombinant nucleotide sequences containing one (or several) coding region(s), this (these) coding region(s) being constituted by:

- a nucleotide sequence coding for a messenger RNA (mRNA), said mRNA itself coding for a lycopene cyclase in plants, or a fragment of said nucleotide sequence, this fragment coding for a mRNA, this mRNA itself coding for a polypeptide having an enzymatic activity equivalent to the one of the lycopene cyclase mentioned above, or a nucleotide sequence derived from the nucleotide sequence mentioned above, or from the fragment mentioned above, particularly by mutation and/or addition and/or suppression and/or substitution of one or several nucleotide(s), this derived sequence coding for a mRNA, this mRNA itself coding for a derived protein having an enzymatic activity equivalent to the one of the lycopene cyclase mentioned above, or

- a nucleotide sequence complementary to the nucleotide sequence coding for a mRNA itself coding for a lycopene cyclase in plants, or to a fragment thereof, or to a derived sequence of these latter, such as defined above, this complementary sequence coding for an antisense mRNA capable of hybridizing with a mRNA such as mentioned above,

for the transformation of plant cells in view of obtaining transgenic plants in which carotenoids biosynthesis is modified either by enhancing or by inhibiting

the production of carotenoids, with respect to the normal contents of carotenoids produced by plants.

The invention relates more particularly to the use, such as mentioned above, of nucleotide sequences containing at least one coding region constituted by:

- the nucleotide sequence represented by SEQ ID NO 1, coding for a mRNA, this mRNA itself coding for the lycopene cyclase represented by SEQ ID NO 2,

- the nucleotide sequence complementary to the one represented by SEQ ID NO 1, this complementary sequence coding for an antisense mRNA capable of hybridizing with the mRNA encoded by the sequence SEQ ID NO 1,

- the nucleotide sequence derived from the sequence SEQ ID NO 1, such as described above, particularly by mutation and/or addition and/or suppression and/or substitution of one or several nucleotide(s), this derived sequence coding for a mRNA itself coding for the lycopene cyclase represented by SEQ ID NO 2, or coding for a derived protein of the said lycopene cyclase, said derived protein having an enzymatic activity equivalent to the one of the said lycopene cyclase in plants,

- the nucleotide sequence derived from the complementary sequence described above, by mutation and/or addition and/or suppression and/or substitution of one or several nucleotide(s), this derived sequence coding for an antisense mRNA capable of hybridizing with the mRNA encoded by the sequence SEQ ID NO 1,

- a fragment of one of the above-mentioned nucleotide sequence, said fragment coding for a mRNA itself coding for a polypeptide having an enzymatic activity equivalent to the one of the lycopene cyclase represented by SEQ ID NO 2, or coding for an antisense mRNA capable of hybridizing with the mRNA encoded by the sequence SEQ ID NO 1.

The present invention also relates to a DNA sequence, containing at least one coding region constituted by:

- the nucleotide sequence represented by SEQ ID NO 1, coding for a mRNA, this mRNA coding itself for the lycopene cyclase represented by SEQ ID NO 2,

- the nucleotide sequence derived from the sequence SEQ ID NO 1, such as described above, particularly by mutation and/or addition and/or suppression and/or substitution of one or several nucleotide(s), this derived sequence coding for a mRNA itself coding for the lycopene cyclase represented by SEQ ID NO 2, or coding for a derived protein of the said lycopene cyclase,

said derived protein having an enzymatic activity equivalent to the one of the said lycopene cyclase in plants,

- a fragment of one of the above-mentioned nucleotide sequence, said fragment coding for a mRNA itself coding for a polypeptide having an enzymatic activity equivalent to the one of the lycopene cyclase represented by SEQ ID NO 2.

The present invention also relates to a DNA sequence containing at least one coding region constituted by:

- the nucleotide sequence complementary to the one represented by SEQ ID NO 1, this complementary sequence coding for an antisense mRNA capable of hybridizing with the mRNA encoded by the sequence SEQ ID NO 1,

- the nucleotide sequence derived from the complementary sequence described above, by mutation and/or addition and/or suppression and/or substitution of one or several nucleotide(s), this derived sequence coding for an antisense mRNA capable of hybridizing with the mRNA encoded by the sequence SEQ ID NO 1,

- a fragment of one of the above-mentioned nucleotide sequence, said fragment coding for an antisense mRNA capable of hybridizing with the mRNA encoded by the sequence SEQ ID NO 1.

The present invention also relates to a mRNA coded by a DNA sequence as defined above, and more particularly coded by the DNA sequence represented by SEQ ID NO 1, with said mRNA being capable of coding itself for the enzyme represented by SEQ ID NO 2, or for a fragment or a protein derived from this enzyme, and presenting an activity which is equivalent to said enzyme in plants.

The present invention also relates to an antisense mRNA comprising nucleotides which are complementary of all or part of the nucleotides constituting a mRNA as defined above, and capable of hybridizing with said mRNA.

The present invention also relates to an antisense mRNA as defined above, characterized by the fact that it is coded by a DNA sequence as defined above, and more particularly by the DNA sequence complementary to the sequence represented by SEQ ID NO 1, and by the fact that it is capable of hybridizing with the mRNA coded by the DNA sequence represented by SEQ ID NO 1.

The present invention also relates to the lycopene cyclase present in *Capsicum annuum* cells and such as represented by SEQ ID NO 2, or any protein derived from said lycopene cyclase, particularly by addition and/or

suppression and/or substitution of one or several amino-acids, or any fragment from said lycopene cyclase or derived sequence, with said fragments and derived sequences being capable of presenting an enzymatic activity equivalent to the one of said lycopene cyclase.

The present invention also relates to a nucleotide sequence coding for the lycopene cyclase represented by SEQ ID NO 2, or any derived sequence or fragment from said lycopene cyclase, as defined above, with said nucleotide sequence being characterized by the fact that it corresponds to all or part of the sequence represented by SEQ ID NO 1, or to any sequence which is derived from this latter by the degeneracy of the genetic code, and being capable of coding for said lycopene cyclase, or a derived sequence, or a fragment from said lycopene cyclase, such as defined above.

In a preferred embodiment, derived nucleotide sequences according to the invention comprise approximately at least 70%, and more particularly approximately at least 80% nucleotides homologous to those of the nucleotide sequence represented by SEQ ID NO 1, or of its complementary sequence.

Advantageously derived proteins according to the invention, comprise approximately at least 50%, and more particularly approximately at least 60% aminoacids homologous to those of the lycopene cyclase represented by SEQ ID NO 2.

Advantageously, nucleotide fragments according to the invention, comprise approximately 100 to approximately 1 000 contiguous nucleotides of the nucleotide sequence represented by SEQ ID NO 1, or of its complementary sequence, or of a derived nucleotide sequence thereof as defined above.

By protein derived from the lycopene cyclase represented by SEQ ID NO 2, or fragment of said lycopene cyclase or of said derived protein, one should understand that it corresponds to polypeptides having a lycopene cyclase activity equivalent to the one of said lycopene cyclase, i.e., polypeptides capable of converting lycopene cyclase to β-carotene. For example, such activity can be measured according to techniques such as described by Cunningham et al., (1994).

The present invention also relates to a complex formed between an antisense mRNA as defined above, and a mRNA as defined above, capable of coding for a lycopene cyclase in plants.

The present invention also relates to a recombinant DNA (also called DNA construct in the following) characterized by the fact that it comprises:

- at least one DNA sequence as defined above, with said sequence being inserted in a heterologous sequence, and being capable of coding for a mRNA

itself capable of coding for a lycopene cyclase or a fragment thereof, or a protein derived from these latter, such as defined above, and/or

- at least one DNA sequence which is complementary of a DNA sequence as defined above, inserted in a heterologous sequence, with said complementary DNA sequence being able to code for an antisense mRNA capable of hybridizing with the mRNA coding for a lycopene cyclase in plants.

The present invention also relates to a DNA recombinant as defined above, characterized by the fact that it comprises the elements necessary to control the expression of the nucleotide sequence as defined above, or of its complementary sequence as defined above, particularly a promoter and a terminator of the transcription of said sequences.

The present invention also relates to a recombinant vector characterized by the fact that it comprises a recombinant DNA as defined above, integrated in one of its sites of its genome, which are non essential for its replication.

The present invention also relates to a process for modifying the production of carotenoid in plants, either by enhancing the production of carotenoid, or by lowering or inhibiting the production of the carotenoid by the plants, with respect to the normal contents of carotenoid produced by plants, said process comprising the transformation of cells of said plants, with a vector as defined above.

The present invention also relates to plants or fragments of plants, particularly fruits, seeds, leaves, petals or cells transformed by incorporation of at least one of the nucleotide sequences as defined above, into their genome.

According to the present invention, there is provided a DNA construct comprising a DNA sequence homologous to some or all of a sequence encoding a lycopene cyclase. The DNA sequence may be derived from cDNA, from genomic DNA or may be synthesized *ab initio*. Preferably, the DNA sequence encodes the lycopene cyclase represented by SEQ ID NO 2.

cDNA clones encoding lycopene cyclase may be obtained from cDNA libraries using standard methods. Sequences coding for the whole, or substantially the whole, of the mRNA produced by the corresponding gene may thus be obtained. The cDNA so obtained may be sequenced according to known methods.

An alternative source of the DNA sequence is a suitable gene encoding the appropriate enzyme. This gene may differ from the corresponding cDNA in that introns may be present. The introns are not transcribed into mRNA (or, if so transcribed, are subsequently cut out). Oligonucleotide probes or the cDNA clone may be used to isolate the lycopene cyclase gene(s) by screening genomic DNA libraries. Such genomic clones may include control sequences operating

in the plant genome. Thus it is also possible to isolate promoter sequences which may be used to drive expression of the enzymes or any other protein. These promoters may be particularly responsive to certain developmental events and environmental conditions. Lycopene cyclase gene promoters may be used to drive expression of any target gene.

A further way of obtaining a lycopene cyclase enzyme DNA sequence is to synthesize it *ab initio* from the appropriate bases, for example using the appropriate cDNA sequence as a guide (for example, SEQ ID NO 1).

It is clear that lycopene cyclase-encoding sequences may be isolated not only from *Capsicum* species but from any suitable plant species. Alternative sources of suitable genes include bacteria, yeast, lower and higher eukaryotes.

The lycopene cyclase-encoding sequences may be incorporated into DNA constructs suitable for plant transformation. These DNA constructs may then be used to modify gene expression in plants. "Antisense" or "partial sense" or other techniques may be used to reduce the expression of the lycopene cyclase(s) in plant tissue. The levels of the lycopene cyclase(s) may also be increased; for example, by incorporation of additional enzyme genes. The additional genes may be designed to give either the same or different spatial and temporal patterns of expression in the plant.

The overall level of lycopene cyclase activity and the relative activities of the individual enzymes affect the development and final form of carotenoid content in the plant and thus determine certain characteristics of the plant parts. Modification of lycopene cyclase activity can therefore be used to modify various aspects of plant (including fruit) quality. The activity levels of the lycopene cyclases may be either reduced or increased during development depending on the characteristics desired for the modified plant. Enhancing expression of a biosynthetic enzyme will increase production of the particular product of bioconversion of the lycopene, i.e. mainly β-carotene and its further derivatives such as zeaxanthin, antheraxanthin, violaxanthin, neoxanthin, capsanthin and capsorubin, and inhibiting expression will decrease such production. Enhancing expression of a degradative enzyme will decrease levels of the lycopene being degraded, while inhibiting expression will increase levels of said lycopene.

For example, the down-regulation of lycopene cyclase activity in peppers (e.g. using antisense or sense constructs) will inhibit β-carotene and its derivatives production to alter fruit colour. Such down-regulation may result in an accumulation of the immediate precursor of the β-carotene which is orange/yellow, i.e. lycopene which is red. Down-regulation of lycopene cyclase may also result in the cyclization of lycopene to produce different

cyclic carotenoid such as δ-carotene or α-carotene and their derivatives. As a further example, over-expression of lycopene cyclase in *Capsicum* species may be used to enhance fruit colour.

Lycopene cyclases may also be expressed in cells, tissues and organisms that do not normally said lycopene cyclases. A DNA sense construct encoding and expressing the functional lycopene cyclase may be used to transform any suitable eukaryotic or prokaryotic cell (plant, fungi, algae, bacteria, animal etc.). If immediate precursor for β-carotene, i.e. lycopene is present in the plant tissue, expression of the enzyme in such tissue leads to β-carotene synthesis. In other cases, the introduction of additional carotenoid biosynthetic genes may be necessary to ensure a supply of the precursor.

DNA constructs according to the invention could be used to produce β-carotene in any higher plant (including *Capsicum* species, tomato, carrot, cabbage, etc.) since the immediate precursor is ubiquitous. This may be useful to change or enhance the colour of the plant or organ depending on the promoter used to drive the production of lycopene cyclase. It is particularly useful for modifying fruit and vegetable colour but may equally be applied to leaves and other organs.

β-carotene produced by a eukaryotic or prokaryotic organism expressing a lycopene cyclase-encoding DNA construct may be extracted for use as a colourant, antioxidant or precursor of vitamin A.

As a further aspect of the invention, we provide a process for the production of β-carotene which comprises transformation of a eukaryotic or prokaryotic cell with a DNA construct encoding and expressing a protein having a lycopene cyclase activity. It may be necessary to transform the cell with additional constructs expressing enzymes needed to produce the necessary precursors.

We further provide a process for the production of lycopene cyclase which comprises transformation of an eukaryotic or prokaryotic cell with a DNA construct encoding at least part of a protein having a lycopene cyclase activity so that production of β-carotene is inhibited.

The activity of the lycopene cyclase may be modified either individually or in combination with modification of the activity of another similar or unrelated enzyme. For example, the activity of the lycopene cyclase may be modified in combination with modification of the activity of a cell wall enzyme involved in fruit ripening.

Use of the novel lycopene cyclase constructs provides a method for modification of plant characteristics comprising modification of the activity of lycopene cyclases.

According to the present invention there is further provided a DNA construct comprising a DNA sequence homologous to some or all of a sequence encoding a lycopene cyclase under the control of a transcriptional initiation region operative in plants, so that the construct can generate RNA in plant cells.

The characteristics of plant parts (particularly fruit) may be modified by transformation with a DNA construct according to the invention. The invention also provides plant cells containing such constructs; plants derived therefrom showing modified fruit characteristics; and seeds of such plants.

A DNA construct according to the invention may be an "antisense" construct generating "antisense" RNA or "sense" construct (encoding at least part of the functional enzyme) generating "sense" RNA. "Antisense RNA" is an RNA sequence which is complementary to a sequence of bases in the corresponding mRNA: complementary in the sense that each base (or the majority of bases) in the antisense sequence (read in the 3' to 5' sense) is capable of pairing with the corresponding base (G with C, A with U) in the mRNA sequence read in the 5' to 3' sense. Such antisense RNA may be produced in the cell by transformation with an appropriate DNA construct arranged to generate a transcript with at least part of its sequence complementary to at least part of the coding strand of the relevant gene (or of a DNA sequence showing substantial homology therewith). "Sense RNA" is an RNA sequence which is substantially homologous to at least part of the corresponding mRNA sequence. Such sense RNA may be produced in the cell by transformation with an appropriate DNA construct arranged in the normal orientation so as to generate a transcript with a sequence identical to at least part of the coding strand of the relevant gene (or of a DNA sequence showing substantial homology therewith). Suitable sense constructs may be used to inhibit gene expression (as described in International Patent Publication WO 91/08299) or to over-express the enzyme.

The constructs of the invention may be inserted into plants to regulate the production of lycopene cyclase. The constructs may be transformed into any dicotyledonous or monocotyledonous plant. Depending on the nature of the construct, the production of the enzyme may be increased or reduced, either throughout or at particular stages in the life of the plant. Generally, as would be expected, production of the enzyme is enhanced only by constructs which express RNA homologous to the substantially complete endogenous enzyme mRNAs. Full-length sense constructs may also inhibit enzyme expression. Constructs containing an incomplete DNA sequence shorter than that corresponding to the complete gene generally inhibit the expression of the gene

and production of the enzymes, whether they are arranged to express sense or antisense RNA.

Full-length antisense constructs also inhibit gene expression.

In a DNA construct according to the invention, the transcriptional initiation region may be derived from any plant-operative promoter. The transcriptional initiation region may be positioned for transcription of a DNA sequence encoding RNA which is complementary to a substantial run of bases in a mRNA encoding the lycopene cyclase (making the DNA construct a full or partial antisense construct).

DNA constructs according to the invention may comprise a base sequence at least 10 bases (preferably at least 35 bases) in length for transcription into RNA. There is no theoretical upper limit to the base sequence - it may be as long as the relevant mRNA produced by the cell - but for convenience it will generally be found suitable to use sequences between 100 and 1000 bases in length. The preparation of such constructs is described in more detail below.

As a source of the DNA base sequence for transcription, a suitable cDNA or genomic DNA or synthetic polynucleotide may be used. The isolation of suitable lycopene cyclase-encoding sequences is described above. Sequences coding for the whole, or substantially the whole, of the appropriate enzyme may thus be obtained. Suitable lengths of these DNA sequences may be cut out for use by means of restriction enzymes. When using genomic DNA as the source of a partial base sequence for transcription it is possible to use either intron or exon regions or a combination of both.

To obtain constructs suitable for expression of the appropriate lycopene cyclase sequence in plant cells, the cDNA sequence as found in the enzyme cDNA or the gene sequence as found in the chromosome of the plant may be used. Recombinant DNA constructs may be made using standard techniques. For example, the DNA sequence for transcription may be obtained by treating a vector containing said sequence with restriction enzymes to cut out the appropriate segment. The DNA sequence for transcription may also be generated by annealing and ligating synthetic oligonucleotides or by using synthetic oligonucleotides in a polymerase chain reaction (PCR) to give suitable restriction sites at each end. The DNA sequence is then cloned into a vector containing upstream promoter and downstream terminator sequences. If antisense DNA is required, the cloning is carried out so that the cut DNA sequence is inverted with respect to its orientation in the strand from which it was cut.

In a construct expressing antisense RNA, the strand that was formerly the template strand becomes the coding strand, and vice versa. The construct will thus encode RNA in a base sequence which is complementary to part or all of the sequence of the enzyme mRNA. Thus the two RNA strands are complementary not only in their base sequence but also in their orientations (5' to 3').

In a construct expressing sense RNA, the template and coding strands retain the assignments and orientations of the original plant gene. Constructs expressing sense RNA encode RNA with a base sequence which is homologous to part or all of the sequence of the mRNA. In constructs which express the functional enzyme, the whole of the coding region of the gene is linked to transcriptional control sequences capable of expression in plants.

For example, constructs according to the present invention may be made as follows. A suitable vector containing the desired base sequence for transcription (such as the lycopene cyclase cDNA clone) is treated with restriction enzymes to cut the sequence out. The DNA strand so obtained is cloned (if desired, in reverse orientation) into a second vector containing the desired promoter sequence and the desired terminator sequence. Suitable promoters include the 35S cauliflower mosaic virus promoter and the tomato polygalacturonase gene promoter sequence (Bird et al., 1988, Plant Molecular Biology, **11:** 651-662) or other developmentally regulated fruit promoters. Suitable terminator sequences include that of the *Agrobacterium tumefaciens* nopaline synthase gene (the nos 3' end).

The transcriptional initiation region (or promoter) operative in plants may be a constitutive promoter (such as the 35S cauliflower mosaic virus promoter) or an inducible or developmentally regulated promoter (such as fruit-specific promoters), as circumstances require. For example, it may be desirable to modify enzyme activity only during fruit development and/or ripening. Use of a constitutive promoter will tend to affect enzyme levels and functions in all parts of the plant, while use of a tissue specific promoter allows more selective control of gene expression and affected functions (e.g. fruit colouration). Thus in applying the invention (for example, to peppers) it may be found convenient to use a promoter that will give expression during fruit development and/or ripening. Thus the antisense or sense RNA is only produced in the organ in which its action is required. Fruit development and/or ripening-specific promoters that could be used include the ripening-enhanced polygalacturonase promoter (International Patent Publication Number WO 92/08798), the E8 promoter (Diekman & Fischer, 1988, EMBO, 7: 3315-3320) and the fruit

specific 2A11 promoter (Pear et al., 1989, Plant Molecular Biology, 13: 639-651).

Carotenoid (particularly β-carotene) content (and hence plant characteristics) may be modified to a greater or lesser extent by controlling the degree of the appropriate lycopene cyclase's sense or antisense mRNA production in the plant cells. This may be done by suitable choice of promoter sequences, or by selecting the number of copies or the site of integration of the DNA sequences that are introduced into the plant genome. For example, the DNA construct may include more than one DNA sequence encoding the lycopene cyclase or more than one recombinant construct may be transformed into each plant cell.

The activity of a first lycopene cyclase may be separately modified by transformation with a suitable DNA construct comprising a DNA sequence encoding the first enzyme. The activity of a second lycopene cyclase may be separately modified by transformation with a suitable DNA construct comprising a DNA sequence encoding the second enzyme. In addition, the activity of both the first and second enzymes may be simultaneously modified by transforming a cell with two separate constructs: the first comprising a first enzyme-encoding sequence and the second comprising a second enzyme-encoding sequence. Alternatively, a plant cell may be transformed with a single DNA construct comprising both a first enzyme-encoding sequence and a second enzyme-encoding sequence.

It is also possible to modify the activity of the lycopene cyclases while also modifying the activity of one or more other enzymes. For example, the other enzymes may be involved in cell metabolism or in fruit development and ripening. Other cell wall metabolising enzymes that may be modified in combination with lycopene cyclases include but are not limited to: pectin esterase, polygalacturonase, β-galactanase, β-glucanase. Other enzymes involved in fruit development and ripening that may be modified in combination with lycopene cyclases include but are not limited to: ethylene biosynthetic enzymes, other carotenoid biosynthetic enzymes including phytoene synthase, carbohydrate metabolism enzymes including invertase.

Several methods are available for modification of the activity of the lycopene cyclases in combination with other enzymes. For example, a first plant may be individually transformed with a lycopene cyclase construct and then crossed with a second plant which has been individually transformed with a construct encoding another enzyme. As a further example, plants may be either consecutively or co-transformed with lycopene cyclase constructs and with appropriate constructs for modification of the activity of the other

enzyme(s). An alternative example is plant transformation with a lycopene cyclase construct which itself contains an additional gene for modification of the activity of the other enzyme(s). The lycopene cyclase constructs may contain sequences of DNA for regulation of the expression of the other enzyme(s) located adjacent to the lycopene cyclase sequences. These additional sequences may be in either sense or antisense orientation as described in International Patent Application Publication number WO 93/23551 (single construct having distinct DNA regions homologous to different target genes). By using such methods, the benefits of modifying the activity of the lycopene cyclase may be combined with the benefits of modifying the activity of other enzymes.

A DNA construct of the invention is transformed into a target plant cell. The target plant cell may be part of a whole plant or may be an isolated cell or part of a tissue which may be regenerated into a whole plant. The target plant cell may be selected from any monocotyledonous or dicotyledonous plant species. Suitable plants include any fruit-bearing plant (such as tomatoes, mangoes, peaches, apples, pears, strawberries, bananas, melons, peppers, chillies, paprika). For any particular plant cell, the lycopene cyclase sequence used in the transformation construct may be derived from the same plant species, or may be derived from any other plant species (sufficient sequence similarity to allow modification of related enzyme gene expression).

Constructs according to the invention may be used to transform any plant using any suitable transformation technique to make plants according to the invention. Both monocotyledonous and dicotyledonous plant cells may be transformed in various ways known to the art. In many cases such plant cells (particularly when they are cells of dicotyledonous plants) may be cultured to regenerate whole plants which subsequently reproduce to give successive generations of genetically modified plants. Any suitable method of plant transformation may be used. For example, dicotyledonous plants such as tomato and melon may be transformed by *Agrobacterium* Ti plasmid technology, such as described by Bevan (1984, Nucleic Acid Research, 12: 8711-8721) or Fillatti et al. (Biotechnology, July 1987, 5: 726-730). Such transformed plants may be reproduced sexually, or by cell or tissue culture.

We further provide a process for modifying the production of carotenoids in plants by transforming such plants with DNA adapted to modify carotenoid biosynthesis and growing such transformed plants or their descendants to produce plant parts (for example leaves, petals or fruit) of modified carotenoid content. Suitable DNA comprises, *inter alia*, constructs according to the present invention, but other similar constructs able to affect the same

carotenoid pathway, such as constructs containing DNA sequences coding for all or part of a capsanthin-capsorubin synthase (CCS), or affecting other parts of the carotenoid pathway may also be used. Such constructs may be adapted to enhance the production of carotenoids (for example β-carotene and its derivatives) or inhibit such production by the plant.

As well as colour production, other important functions may be modified by the process of the invention. Thus β-carotene (a precursor of Vitamin A) and other carotenoids are important to human health, and have been claimed to have a protective effect against certain diseases. More particularly, Vitamin A is known as a radical scavenger which can be useful as protectors against free radicals and thus be used in the frame of the prevention or the treatment of diseases caused by free radicals, such as certain type of cancer. Food plants may be modified by transformation with the constructs of the invention so that they have a higher content of such compounds: or other plants may be so modified, so that they can act as a source from which such compounds can be extracted.

In this respect, the present invention relates more particularly to a process for enhancing the production of carotenoids, and more particularly of β-carotene (provitamin A) and thus of Vitamin A with respect to the normal contents of Vitamin A produced by plants, said process comprising the transformation of cells of said plants with a vector as defined above, more particularly with a vector comprising a DNA sequence coding, via a sense mRNA, for a lycopene cyclase or for a derived protein or for fragments thereof as defined above.

The invention relates more particularly to plants or part of plants, seeds and fruits, genetically transformed with a DNA sequence according to the invention, and comprising Vitamin A at a higher level than the normal content of Vitamin A, if any, produced by these plants.

Among transgenic plants containing higher levels of Vitamin A according to the invention, one can cite tomato fruits, and potato tubers.

The present invention also more particularly to a process for inhibiting the production of carotenoids, and more particularly of β-carotene (provitamin A) and thus of Vitamin A with respect to the normal contents of Vitamin A produced by plants, said process comprising :

- either the transformation of cells of said plants with a vector as defined above, more particularly with a vector comprising a DNA sequence coding, via a sense mRNA, for a lycopene cyclase or for a derived protein or for fragments thereof as defined above ; the inhibition of the the carotenoids will then proceed via a mechanism of co-suppression,

- or the transformation of cells of said plants with a vector as defined above, more particularly with a vector comprising a DNA sequence coding for an antisense mRNA as defined above and capable of hybridizing with a mRNA coding for a lycopene cyclase in plants or for a derived protein or for fragments thereof as defined above

The invention relates more particularly to plants or part of plants, seeds and fruits, genetically transformed with a DNA sequence according to the invention, and which do not comprise carotenoids, or comprising carotenoids, and more particularly Vitamin A, at a lower level than the normal content of Vitamin A, if any, produced by these plants.

Carotenoids are also believed to have a role in protecting plants against high light intensity damage, so plants with a higher content of such compounds may be of value in combating the effects of any global climate change.

In this way, plants can be generated which have modified colour due to promotion or inhibition of the pathways of carotenoid biosynthesis. In particular, lycopene cyclase constructs may be used to promote or inhibit the production of the orange/yellow colour associated with β-carotene. For example, inhibition of this red colour in peppers (e.g. by transformation with antisense or sense constructs) may give fruit of an attractive shade of red. Promotion of β-carotene production (e.g. by sense over-expression constructs) may produce peppers of orange/yellow colour, or of a colour determined by a β-carotene derivative such as a deeper red colour, due to the biosynthesis of capsorubin or capsanthin, which may appear more appetising to the consumer.

The invention may also be used to introduce a specific colour into parts of plants other than the fruit. For example, promotion of β-carotene may be brought about by inserting one or more functional copies of the gene cDNA, or of the full-length gene, under control of a promoter functional in plants. If β-carotene is naturally expressed in the plant, the promoter may be selected to give a higher degree of expression than is given by the natural promoter.

Examples of genetically modified plants according to the present invention include fruit-bearing plants. The fruit of such plants may be made more attractive (or at least interesting) by inducing or intensifying a specific colour therein. Other plants that may be modified by the process of the invention include tubers such as radishes, turnips and potatoes, as well as cereals such as maize (corn), wheat, barley and rice. Flowers of modified colour, and ornamental grasses either red or reddish overall, or having red seedheads, may be produced.

As already discussed, plants produced by the process of the invention may also contain other recombinant constructs, for example constructs having

other effects on fruit ripening. For example fruit of enhanced colour according to the invention may also contain constructs inhibiting the production of enzymes such as polygalacturonase and pectinesterase, or interfering with ethylene production. Fruit containing both types of recombinant construct may be made either by successive transformations, or by crossing two varieties that each contain one of the constructs, and selecting among the progeny for those that contain both.

The invention is further illustrated in the detailed description which follows of the cloning and sequencing of the cDNA encoding a lycopene cyclase in *C. annuum*.

MATERIALS AND METHODS

Materials, Pepper *(Capsicum annuum*, cv. Yolo Wonder) plants were grown under greenhouse conditions. For RNA isolation, plant materials were harvested between 9:00 and 10:00 a.m and immediately frozen in liquid nitrogen. The *Arabidopsis thaliana* cDNA clone ATTS2157 was obtained from Dr . M. Caboche and co-workers (INRA Versailles, France)

Cloning of cDNAs. A *C. annuum* cDNA library prepared in λgt11 from poly (A^+) RNA isolated from a fruit at an early ripening stage (Kuntz et al., 1992) was screened using radiolabelled probes. DNA fragments used as probes were isolated from low-melting temperature agarose and random-primed labelled using standard techniques in the presence of [^{32}P]dCTP.

Hybridizations and washes were performed in 2xSSC at either 60°C or 50°C. For stringent conditions the hybridization and wash temperatures were 65°C (in 02xSSC for the washes).

Subcloning and sequencing. Subcloning of DNA in pBluescript KS^- was performed as described previously (Kuntz et al., 1992). Sequencing was performed either manually (Zhang et al. 1988) or using an automated Applied sequencer. DNA sequence analysis was performed using the programs of the University of Wisconsin Genetics Computer Group. Search through the sequence databases used the National Center for Biotechnology Information server (NCBI, Blast Programs).

RNA gel blot analysis. Total RNA (10μg) were separated on formaldehyde-containing agarose gels and blotted onto nitrocellulose. Two subclones of the *C. annuum* lycopene cyclase cDNA inserted in pBluescript KS^- were used to generate radiolabelled riboprobes by the T3 RNA polymerase

in the presence of [^{32}P]UTP, cold ATP, CTP, and GTP. These riboprobes correspond to the first 542 and last 573 nucleotides, respectively of the complete transcript. Hybridizations were performed using the above-mentioned stringent conditions.

Expression in *E. coli*.

E. coli strains were grown in the presence of the appropriate antibiotics and chlorophenyl-triethylamine (CPTA) at 40 µM or IPTG at 40µM when mentioned. Plasmid pACYC-EBI is a derivative of pACYC184 harboring the *Erwinia uredovora crtE*, *crtB*, and *crtI* genes. A JM101 strain containing pACYC-EBI (chloramphenicol^{R}) was obtained from Prof. G. Sandmann and co-workers (University of Frankfurt, Germany) and used as the recipient for cDNAs inserted in pBluescript KS^{-} (ampicillin ^{R}) in the sense orientation with respect to the *lac*Z promoter.

HPLC analysis of pigments

10 ml cultures of *E. coli* cells were grown in darkness overnight in LB medium. After centrifugation, the bacterial pellet was resuspended in 1 ml of acetone. The samples were incubated at 65°C for 30 min, centrifuged at 10 000 g and the supernatants were analyzed using a Waters HPLC system equipped with a 250/8/4 Nucleosil 5 C18 column (Macherey-Nagel). Eluent was 100% acetonitrile and peaks were detected at 450 nm by a Waters diode-array detector. Carotenoids were identified by their retention time and their typical absorption spectra.

Results

cDNA cloning

The partial sequence of an expressed sequence tag (EST) from *Arabidopsis thaliana* (deposited in the databank under the locus name ATTS2157; Desprez et al., 1994) shares significant sequence similarity at the amino acid level with the previously reported *C. annuum* capsanthin/capsorubin synthase (CCS) (Bouvier et al., 1994). Since a CCS activity is unlikely to exist in *A. thaliana*, this observation suggests that EST-ATTS2157 may correspond to a cDNA encoding a related enzyme of the carotenoid biosynthetic pathway.

Therefore, it has been decided to clone the corresponding cDNA from a *C. annuum* ripening fruit library using EST-ATTS2157 as a hybridization probe. Numerous positive plaques were obtained at hybridization temperatures

of 50°C and 60°C. However, several plaques showed a higher relative hybridization signal at 60°C vs. 50°C, when compared to the signal produced by most of the other positive plaques. Control experiments (data not shown) revealed that the plaques hybridizing weakly at 60°C to EST-ATTS2157 hybridized to the CCS cDNA in stringent conditions. In contrast, the plaques hybridizing strongly to EST-ATTS2157 at 60°C did not hybridize to CCS in stringent conditions. One of the latter clones was further purified and its ca. 500 bp was subcloned in a plasmid vector and then used to isolate the corresponding full-length clone by hybridization under stringent conditions.

Out of approximately $2x10^5$ clones from the cDNA library, 10 positive clone were obtained. After further plaque purification, 4 clones showing the largest inserts were subcloned in a plasmid vector and sequenced. The shorter cDNAs correspond to truncated transcripts and did not show sequence difference. The original 500 bp cDNA corresponds to the 3'-end portion of the larger cDNA.

Amino acid sequence comparison

The amino acid sequence deduced from the cloned cDNA is 498 residue long. This sequence is likely to be a full-length one since stop codons are found in frame upstream of the open reading frame. The calculated MW of the encoded precursor polypeptide is 55.6 kDa.

When aligned with the CCS sequence, an overall identity of 55 % (72% similarity) was found. Little sequence identity was observed in the NH_2-portion of the precursor proteins. This is a normal feature of transit peptides for plastid targeting of precursor polypeptides. These presequences are usually less conserved than the mature polypeptides. Moreover, usual features of transit peptides (e.g. presence of numerous hydroxylated or positively charged amino acids) are found in the 56 first amino acid sequence. In addition, comparison to the CCS transit peptide suggest that post-translocation cleavage occurs before the acidic region starting at position 57 (most likely in the region of residue 47 and 54).

Consequently, the calculated MW of the mature polypeptide is ca. 50 kDa. Its pI is 6.2. Its sequence identity with the mature CCS is 55.6 %. Like in several enzymes of the carotenoid biosynthetic pathway (for a review see Sandmann, 1994) a potential dinucleotide binding site is present near the NH_2-end of the mature polypeptide.

In addition to this motif, the mature polypeptide contains two conserved motifs I and II also found in the *Erwinia uredovora* and *E. herbicola* lycopene cyclases (Misawa et al., 1990, Hundle et al., 1994).

The overall identity with these bacterial lycopene cyclases (when numerous gaps were introduced to optimize identity) is 23 % (52 % similarity). Furthermore, when the sequence reported here was compared to the recently published sequence (Cunningham et al., 1994) of a cyanobacterial (*Synechococcus*) lycopene cyclase, an overall identity of 35 % (56 % similarity) was obtained. Alignment of the motifs I and II with the corresponding regions of the *Erwinia* lycopene cyclases shows that both motifs resemble each other and that such a motif is also present in the *Erwinia* β-carotene hydroxylase.

Taken together, these observations suggest that the cloned cDNA encodes a plant lycopene cyclase (tentatively termed CrtL).

Expression of the cDNA in *E. coli*

In order to confirm that the cloned cDNA encodes a lycopene cyclase, expression assays were performed in *E. coli*. Plasmids containing the full-length cDNA were introduced in an *E. coli* strain containing plasmid pACYC-EBI. This plasmid harbors *Erwinia uredovora* genes for geranylgeranyl pyrophosphate synthase, phytoene synthase and phytoene desaturase (Misawa et al., 1990). Consequently, this *E. coli* strain accumulates lycopene (cells have a pinkish colour). After transformation with the crtL cDNA, yellow colonies were formed.

To identify the carotenoids which were formed, HPLC analysis was performed. As expected, the elution profile of the pigments extracted from pACYC-EBI-containing cells shows a single peak which has the retention time of a lycopene standard. In the extract from the strain expressing in addition CrtL, this lycopene peak was absent and a new peak appeared, which has the retention time and absorption spectrum of a β-carotene standard. The same profile was obtained in the presence or absence of IPTG (an inducer of the lacZ promoter which is driving expression of the cDNA) in the growth medium, indicating that sufficient enzyme activity was produced in both cases to convert 100% of lycopene to β-carotene (see Figure 1).

Expression pattern of the lycopene cyclase gene during plant development

RNA gel blot analysis was performed using total RNA isolated from *C. annuum* leaves and fruits at various development stages. In order to avoid cross-hybridization to CCS transcripts, two subfragments of the lycopene cyclase cDNA (from the 5'-end and 3'-end regions) were radiolabelled (see Materials and Methods).

Only weak hybridization signals could be seen after long exposure of the autoradiograph. This observation, as well as the low abundance of this clone in the cDNA library, indicate that lycopene cyclase is encoded by a minor transcript in *C. annuum* and that this transcript is significantly less abundant than the CCS transcript for instance.

The lycopene cyclase transcript was detected at all stages of leaf fruit development. Unlike CCS, no significant increase in transcript level was observed during fruit ripening. The lycopene cyclase transcript level was approximately five time higher in young leaves than in senescing leaves and fruits.

Discussion

The availability of molecular clones for carotenoid biosynthetic enzymes from plants (for a review see Bartley et al., 1994) represents an important breakthrough in the study of this biosynthetic pathway. In the case of lycopene cyclisation, comparison of bacterial gene sequences have shown previously that the enzymes involved in β-carotene synthesis are of different types in non-photosynthetic bacteria (Misawa et al., 1990; Hundle et al., 1994) and in a cyanobacteria (Cunnigham et al., 1994). In this report we show that a *C. annuum* chromoplast enzyme which catalyzes the conversion of lycopene to β-carotene (when its cDNA is expresses in *E. coli),* is more closely related to the cyanobacterial lycopene cyclase (35% sequence identity). However, this sequence identity is lower than the one shared for example by phytoene desaturases from the same organisms (65% identity). It therefore appears that the enzymatic conversion of lycopene to β-carotene can tolerate extensive sequence variability within the relevant enzymes.

It also appeared that the *C. annuum* lycopene cyclase is more closely related (55% identity) to a *C. annuum* enzyme which is involved in the conversion of the epoxy-carotenoids antheraxanthin and violaxanthin in the keto-carotenoids capsanthin and capsorubin, respectively (Bouvier et al., 1994). When expressed in *E. coli* the latter enzyme was found to also possess a lycopene cyclase activity. Therefore, it can be postulated that the massive and specific channelling of linear carotenoids into the β-carotene pathway in red *C. annuum* fruits is due to the concomitant action of lycopene β-cyclase and CCS.

Alignment of these sequences shows the presence of a typical dinucleotide-binding site which has been suggested to bind FAD in the cyanobacterial enzyme (Cunnigham et al., 1994). Two other conserved motifs, which are related to each other, are also found (Fig. 1B). These three

sequences being the only one to be highly conserved, it seems likely that they are of central importance in the catalytic reaction. In addition, two conserved cysteines are found (position 177 and 344) which could be responsible for a sensitivity of lycopene cyclase to sulfhydryl reagents (Camara and Dogbo,1986).

The sequence conservation between lycopene cyclase and CCS, and the fact that the latter enzyme has also a lycopene cyclase activity is likely to be related to similarities in the chemical mechanisms leading to the formation of β-rings in β-carotene and κ-rings in capsanthin and capsorubin. The proposed mechanisms for both reactions occur via similar carbocation intermediates. In addition, both reactions are likely to be initiated by a protonic attack on either a double bond or an epoxy group.

The striking sequence identity observed between lycopene cyclase and CCS from *C. annuum* strongly suggest that both genes originated from a common ancestral gene. Taken together these data suggest that the species-specific gene encoding CCS has arisen from duplication and mutation of a candidate for such an ancestral gene, although it cannot be excluded from the present state of our knowledge that this ancestral gene was in fact encoding an enzyme catalyzing a different but chemically related reaction such as α-carotene or neoxanthin synthesis. These data provide for the first time an explanation at the molecular level for the diversity of carotenoids in plants, and in particular for the origin of species-specific carotenoids.

Legend to Figure 1

HPLC elution profiles and absorption spectra of pigments extracted from *E. coli* cells producing lycopene and expressing plant cDNA.

Figure 1A. Elution profiles of control cultures containing pACYC-EBI (expressing the *E. uredovora* genes *crt-EBI*) and of cultures expressing in addition *C. annumm* CrtL or CCS cDNAs. Peaks 1 and 1' have the retention time of a lycopene standard. Peaks 2 and 2' have the retention time of a β-carotene standard.

Figure 1B. Typical absorption spectrum of peaks 1 and 1'.

Figure 1C. Typical absorption spectrum of peaks 2 and 2'.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:
(i) APPLICANT:
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(B) STREET: 3, rue Michel-Ange
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(ii) TITLE OF INVENTION: DNA SEQUENCES ENCODING A LYCOPENE CYCLASE, ANTISENSE SEQUENCES DERIVED THEREFROM AND THEIR USE FOR THE MODIFICATION OF CAROTENOIDS LEVELS IN PLANTS

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1942 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 266..1759

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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CTTAATTATA GAAATACTTA AGATATATCA TTGCCCTTTA ATCATTTATT TTTAACTCTT      60

TTAAGTGTTT AAAGATTGAT TCTTTGTACA TGTTCTGCTT CATTTGTGTT GAAAATTGAG     120

TTGTTTTCTT GAATTTTGCA AGAATATAGG GGACCCCATT TGTGTTGAAA ATTGAGCAGC     180

TTTCTTTGTG TTTTGTTCGA TTTTTCAAGA ATATAGGACC CCATTTTCTG TTTTCTTGAG     240

ATAAATTGCA CCTTGTTGGG AAAAT ATG GAT ACG CTC TTG AGA ACC CCA AAC       292
                           Met Asp Thr Leu Leu Arg Thr Pro Asn
                             1               5

AAT CTT GAA TTT CTG CAT GGA TTT GGT GTT AAA GTT AGT GCC TTT AGC       340
Asn Leu Glu Phe Leu His Gly Phe Gly Val Lys Val Ser Ala Phe Ser
 10                  15                  20                  25

TCT GTG AAG TCT CAG AAG TTT GGT GCT AAG AAG TTT TGT GAA GGT TTG       388
Ser Val Lys Ser Gln Lys Phe Gly Ala Lys Lys Phe Cys Glu Gly Leu
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GGG AGT AGA AGT GTC TGT GTG AAG GCT AGT AGT AGT GCT CTT TTG GAG      436
Gly Ser Arg Ser Val Cys Val Lys Ala Ser Ser Ser Ala Leu Leu Glu
            45                  50                  55

CTT GTA CCT GAG ACA AAA AAG GAA AAT CTT GAT TTT GAG CTT CCT ATG      484
Leu Val Pro Glu Thr Lys Lys Glu Asn Leu Asp Phe Glu Leu Pro Met
        60                  65                  70

TAT GAC CCT TCA AAA GGG GTT GTT GTG GAT CTT GCT GTG GTC GGT GGT      532
Tyr Asp Pro Ser Lys Gly Val Val Val Asp Leu Ala Val Val Gly Gly
    75                  80                  85

GGT CCT GCA GGT CTT GCT GTT GCA CAG CAA GTT TCT GAA GCA GGA CTT      580
Gly Pro Ala Gly Leu Ala Val Ala Gln Gln Val Ser Glu Ala Gly Leu
90                  95                  100                 105

TCT GTT TGT TCG ATT GAT CCG AAT CCT AAA TTG ATA TGG CCT AAT AAC      628
Ser Val Cys Ser Ile Asp Pro Asn Pro Lys Leu Ile Trp Pro Asn Asn
                110                 115                 120

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Tyr Gly Val Trp Val Asp Glu Phe Glu Ala Met Asp Leu Leu Asp Cys
            125                 130                 135

CTT GAT GCT ACT TGG TCT GGT GCA GCG GTG TAC ATT GAT GAT AAA ACA      724
Leu Asp Ala Thr Trp Ser Gly Ala Ala Val Tyr Ile Asp Asp Lys Thr
        140                 145                 150

ACT AAA GAT CTT AAT AGA CCT TAT GGA AGG GTT AAC CGA AAG CAG TTG      772
Thr Lys Asp Leu Asn Arg Pro Tyr Gly Arg Val Asn Arg Lys Gln Leu
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Lys Ser Lys Met Met Gln Lys Cys Ile Leu Asn Gly Val Lys Phe His
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Ser Leu Val Ala Arg Pro Gly Leu Gly Met Asp Asp Ile Gln Glu Arg
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ATG GTG GCT CGT TTA AGT CAC TTG GGG ATA AAA GTT AAG AGC ATT GAA      1252
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    315                 320                 325

GAG GAT GAA CAT TGT GTA ATA CCA ATG GGT GGT CCT CTT CCA GTA TTA      1300
Glu Asp Glu His Cys Val Ile Pro Met Gly Gly Pro Leu Pro Val Leu
330                 335                 340                 345

CCT CAG AGA GTT GTT GGA ATT GGT GGC ACA GCC GGT ATG GTT CAT CCA      1348
Pro Gln Arg Val Val Gly Ile Gly Gly Thr Ala Gly Met Val His Pro
                350                 355                 360

TCC ACC GGT TAT ATG GTA GCA AGG ACA CTA GCT GCA GCT CCT GTC GTT      1396
Ser Thr Gly Tyr Met Val Ala Arg Thr Leu Ala Ala Ala Pro Val Val
            365                 370                 375

GCC AAT GCC ATA ATT CAG TAC CTC AGT TCT GAA AGA AGT CAT TCG GGT      1444
Ala Asn Ala Ile Ile Gln Tyr Leu Ser Ser Glu Arg Ser His Ser Gly
        380                 385                 390

GAT GAG TTA TCC GCA GCT GTT TGG AAG GAT TTG TGG CCG ATA GAG AGG      1492
Asp Glu Leu Ser Ala Ala Val Trp Lys Asp Leu Trp Pro Ile Glu Arg
    395                 400                 405

AGG CGT CAA AGA GAG TTC TTC TGC TTC GGT ATG GAC ATT CTT CTG AAG      1540
Arg Arg Gln Arg Glu Phe Phe Cys Phe Gly Met Asp Ile Leu Leu Lys
410                 415                 420                 425

CTT GAC TTA CCG GCT ACA AGG AGG TTC TTT GAT GCA TTC TTC GAC TTA      1588
Leu Asp Leu Pro Ala Thr Arg Arg Phe Phe Asp Ala Phe Phe Asp Leu
                430                 435                 440

GAA CCT CGT TAT TGG CAT GGC TTC TTG TCA TCC AGG TTG TTT CTA CCT      1636
Glu Pro Arg Tyr Trp His Gly Phe Leu Ser Ser Arg Leu Phe Leu Pro
            445                 450                 455

GAA CTC ATA GTT TTT GGG CTC TCA CTT TTC TCT CAT GCT TCA AAT ACT      1684
Glu Leu Ile Val Phe Gly Leu Ser Leu Phe Ser His Ala Ser Asn Thr
        460                 465                 470

TCT AGA TTA GAG ATA ATG ACA AAG GGA ACT CTT CCA TTA GTA CAT ATG      1732
Ser Arg Leu Glu Ile Met Thr Lys Gly Thr Leu Pro Leu Val His Met
    475                 480                 485

ATC AAC AAT TTG TTA CAG GAT AAA GAA TGAATTCGAC TTATCTGGGA           1779
Ile Asn Asn Leu Leu Gln Asp Lys Glu
490                 495
```

TCTTGTATCA CAGTCTTAAT TATAGAAATA CTTAAGATAT ATCATTGCCC TTAATCATT 1839

TATTTTTAAC TCTTTTAAGT GTTTAAAGAT TGATTCTTTG TACATGTTCT GCTTCATTTG 1899

TGTTGAAAAT TGAGTTGTTT TCCTTCGTCA TTCATCATCC ATC 1942

(2) INFORMATION FOR SEQ ID NO: 2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 498 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Asp Thr Leu Leu Arg Thr Pro Asn Asn Leu Glu Phe Leu His Gly
1 5 10 15

Phe Gly Val Lys Val Ser Ala Phe Ser Ser Val Lys Ser Gln Lys Phe
20 25 30

Gly Ala Lys Lys Phe Cys Glu Gly Leu Gly Ser Arg Ser Val Cys Val
35 40 45

Lys Ala Ser Ser Ser Ala Leu Leu Glu Leu Val Pro Glu Thr Lys Lys
50 55 60

Glu Asn Leu Asp Phe Glu Leu Pro Met Tyr Asp Pro Ser Lys Gly Val
65 70 75 80

Val Val Asp Leu Ala Val Val Gly Gly Gly Pro Ala Gly Leu Ala Val
85 90 95

Ala Gln Gln Val Ser Glu Ala Gly Leu Ser Val Cys Ser Ile Asp Pro
100 105 110

Asn Pro Lys Leu Ile Trp Pro Asn Asn Tyr Gly Val Trp Val Asp Glu
115 120 125

Phe Glu Ala Met Asp Leu Leu Asp Cys Leu Asp Ala Thr Trp Ser Gly
130 135 140

Ala Ala Val Tyr Ile Asp Asp Lys Thr Thr Lys Asp Leu Asn Arg Pro
145 150 155 160

Tyr Gly Arg Val Asn Arg Lys Gln Leu Lys Ser Lys Met Met Gln Lys
165 170 175

Cys Ile Leu Asn Gly Val Lys Phe His Gln Ala Lys Val Ile Lys Val
180 185 190

Ile His Glu Glu Ser Lys Ser Met Leu Ile Cys Asn Asp Gly Ile Thr
195 200 205

Ile Gln Ala Thr Val Val Leu Asp Ala Thr Gly Phe Ser Arg Ser Leu
210 215 220

Val Gln Tyr Asp Lys Pro Tyr Asn Pro Gly Tyr Gln Val Ala Tyr Gly
225 230 235 240

```
Ile Leu Ala Glu Val Glu Glu His Pro Phe Asp Val Asn Lys Met Val
                245                 250                 255

Phe Met Asp Trp Arg Asp Ser His Leu Lys Asn Asn Val Glu Leu Lys
            260                 265                 270

Glu Arg Asn Ser Arg Ile Pro Thr Phe Leu Tyr Ala Met Pro Phe Ser
        275                 280                 285

Ser Asn Arg Ile Phe Leu Glu Glu Thr Ser Leu Val Ala Arg Pro Gly
    290                 295                 300

Leu Gly Met Asp Asp Ile Gln Glu Arg Met Val Ala Arg Leu Ser His
305                 310                 315                 320

Leu Gly Ile Lys Val Lys Ser Ile Glu Glu Asp Glu His Cys Val Ile
                325                 330                 335

Pro Met Gly Gly Pro Leu Pro Val Leu Pro Gln Arg Val Val Gly Ile
            340                 345                 350

Gly Gly Thr Ala Gly Met Val His Pro Ser Thr Gly Tyr Met Val Ala
        355                 360                 365

Arg Thr Leu Ala Ala Ala Pro Val Val Ala Asn Ala Ile Ile Gln Tyr
    370                 375                 380

Leu Ser Ser Glu Arg Ser His Ser Gly Asp Glu Leu Ser Ala Ala Val
385                 390                 395                 400

Trp Lys Asp Leu Trp Pro Ile Glu Arg Arg Arg Gln Arg Glu Phe Phe
                405                 410                 415

Cys Phe Gly Met Asp Ile Leu Leu Lys Leu Asp Leu Pro Ala Thr Arg
            420                 425                 430

Arg Phe Phe Asp Ala Phe Phe Asp Leu Glu Pro Arg Tyr Trp His Gly
        435                 440                 445

Phe Leu Ser Ser Arg Leu Phe Leu Pro Glu Leu Ile Val Phe Gly Leu
    450                 455                 460

Ser Leu Phe Ser His Ala Ser Asn Thr Ser Arg Leu Glu Ile Met Thr
465                 470                 475                 480

Lys Gly Thr Leu Pro Leu Val His Met Ile Asn Asn Leu Leu Gln Asp
                485                 490                 495

Lys Glu
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CLAIMS

1. Use of recombinant nucleotide sequences containing one (or several) coding region(s), this (these) coding region(s) being constituted by:

- a nucleotide sequence coding for a messenger RNA (mRNA), said mRNA itself coding for a lycopene cyclase in plants, or a fragment of said nucleotide sequence, this fragment coding for a mRNA, this mRNA itself coding for a polypeptide having an enzymatic activity equivalent to the one of the lycopene cyclase mentioned above, or a nucleotide sequence derived from the nucleotide sequence mentioned above, or from the fragment mentioned above, particularly by mutation and/or addition and/or suppression and/or substitution of one or several nucleotide(s), this derived sequence coding for a mRNA, this mRNA itself coding for a derived protein having an enzymatic activity equivalent to the one of the lycopene cyclase mentioned above, or

- a nucleotide sequence complementary to the nucleotide sequence coding for a mRNA itself coding for a lycopene cyclase in plants, or to a fragment thereof, or to a derived sequence of these latter, such as defined above, this complementary sequence coding for an antisense mRNA capable of hybridizing with a mRNA such as mentioned above,

for the transformation of plant cells in view of obtaining transgenic plants in which carotenoids biosynthesis is modified either by enhancing or by inhibiting the production of carotenoids, with respect to the normal contents of carotenoids produced by plants.

2. Use of recombinant nucleotide sequences according to claim 1, characterized in that they contain at least one coding region, constituted by:

- the nucleotide sequence represented by SEQ ID NO 1, coding for a mRNA, this mRNA itself coding for the lycopene cyclase represented by SEQ ID NO 2,

- the nucleotide sequence complementary to the one represented by SEQ ID NO 1, this complementary sequence coding for an antisense mRNA capable of hybridizing with the mRNA encoded by the sequence SEQ ID NO 1,

- the nucleotide sequence derived from the sequence SEQ ID NO 1, such as described above, particularly by mutation and/or addition and/or suppression and/or substitution of one or several nucleotide(s), this derived sequence coding for a mRNA itself coding for the lycopene cyclase represented by SEQ ID NO 2, or coding for a derived protein of the said lycopene cyclase,

said derived protein having an enzymatic activity equivalent to the one of the said lycopene cyclase in plants,

- the nucleotide sequence derived from the complementary sequence described above, by mutation and/or addition and/or suppression and/or substitution of one or several nucleotide(s), this derived sequence coding for an antisense mRNA capable of hybridizing with the mRNA encoded by SEQ ID NO 1,

- a fragment of one of the above-mentioned nucleotide sequence, said fragment coding for a mRNA itself coding for a polypeptide having an enzymatic activity equivalent to the one of the lycopene cyclase represented by SEQ ID NO 2, or coding for an antisense mRNA capable of hybridizing with the mRNA encoded by the sequence SEQ ID NO 1.

3. DNA sequence, containing at least one coding region constituted by:

- the nucleotide sequence represented by SEQ ID NO 1, coding for a mRNA, this mRNA coding itself for the lycopene cyclase represented by SEQ ID NO 2,

- the nucleotide sequence derived from the sequence SEQ ID NO 1, such as described above, particularly by mutation and/or addition and/or suppression and/or substitution of one or several nucleotide(s), this derived sequence coding for a mRNA itself coding for the lycopene cyclase represented by SEQ ID NO 2, or coding for a derived protein of the said lycopene cyclase, said derived protein having an enzymatic activity equivalent to the one of the said lycopene cyclase in plants,

- a fragment of one of the above-mentioned nucleotide sequence, said fragment coding for a mRNA itself coding for a polypeptide having an enzymatic activity equivalent to the one of the lycopene cyclase represented by SEQ ID NO 2.

4. DNA sequence, containing at least one coding region constituted by:

- the nucleotide sequence complementary to the one represented by SEQ ID NO 1, this complementary sequence coding for an antisense mRNA capable of hybridizing with the mRNA encoded by the sequence SEQ ID NO 1,

- the nucleotide sequence derived from the complementary sequence described above, by mutation and/or addition and/or suppression and/or substitution of one or several nucleotide(s), this derived sequence coding for an antisense mRNA capable of hybridizing with one of the mRNA mentioned above,

- a fragment of one of the above-mentioned nucleotide sequence, said fragment coding for a mRNA itself coding for an antisense mRNA capable of hybridizing with the mRNA encoded by the sequence SEQ ID NO 1.

5. mRNA coded by a DNA sequence according to claim 3, and more particularly coded by the DNA sequence represented by SEQ ID NO 1, with said mRNA being capable of coding itself for the lycopene cyclase represented by SEQ ID NO 2, or for a fragment or a protein derived from this enzyme, and presenting an activity which is equivalent to said enzyme in plants.

6. Antisense mRNA comprising nucleotides which are complementary of all or part of the nucleotides constituting a mRNA according to claim 5, and capable of hybridizing with said mRNA.

7. Antisense mRNA according to claim 6, characterized by the fact that it is coded by a DNA sequence according to claim 4, and by the fact that it is capable of hybridizing with the mRNA coded by the DNA sequence represented by SEQ ID NO 1.

8. Lycopene cyclase present in *Capsicum annuum* cells and such as represented by SEQ ID NO 2, or any protein derived from said lycopene cyclase, particularly by addition and/or suppression and/or substitution of one or several amino-acids, or any fragment from said lycopene cyclase or derived sequence, with said fragments and derived sequences being capable of presenting an enzymatic activity equivalent to the one of said lycopene cyclase.

9. Nucleotide sequence coding for the lycopene cyclase represented by SEQ ID NO 1, or any derived sequence or fragment from said lycopene cyclase, according to claim 8, with said nucleotide sequence being characterized by the fact that it corresponds to all or part of the sequence represented by SEQ ID NO 1, or to any sequence which is derived from this latter by the degeneracy of the genetic code, and being capable of coding for the lycopene cyclase, or a derived sequence, or a fragment from said lycopene cyclase, such as defined in claim 8.

10. Complex formed between an antisense mRNA according to claim 6 or 7, and a mRNA according to claim 3, capable of coding for a lycopene cyclase in plants.

11. Recombinant DNA characterized by the fact that it comprises

- a DNA sequence according to claim 3, with said sequence according to claim 3 being inserted in a heterologous sequence and being capable of coding for mRNA itself capable of coding for a lycopene cyclase, and/or a fragment thereof, or a protein derived from these latter, or

- a DNA sequence which is complementary of a DNA sequence according to claim 3, inserted in a heterologous sequence, with said complementary DNA sequence being able to code for an antisense mRNA capable of hybridizing with the mRNA coding for a lycopene cyclase in plants.

12. DNA recombinant according to claim 11, characterized by the fact that it comprises the elements necessary to control the expression of the nucleotide sequence according to claim 3, or of its complementary sequence according to claim 4, particularly a promoter and a terminator of the transcription of said sequences.

13. Recombinant vector characterized by the fact that it comprises a recombinant DNA according to claims 11 or 12, integrated in one of its sites of its genome, which are non essential for its replication.

14. Process for modifying the production of carotenoid in plants, either by enhancing the production of carotenoid, or by lowering or inhibiting the production of the carotenoid by the plants, with respect to the normal contents of carotenoid produced by plants, said process comprising the transformation of cells of said plants, with a vector according to claim 13.

15. Plants or fragments of plants, particularly fruits, seeds, leaves, petals or cells transformed by incorporation of at least one of the nucleotide sequences according to claim 3 or 4, into their genome.

Figure 1A

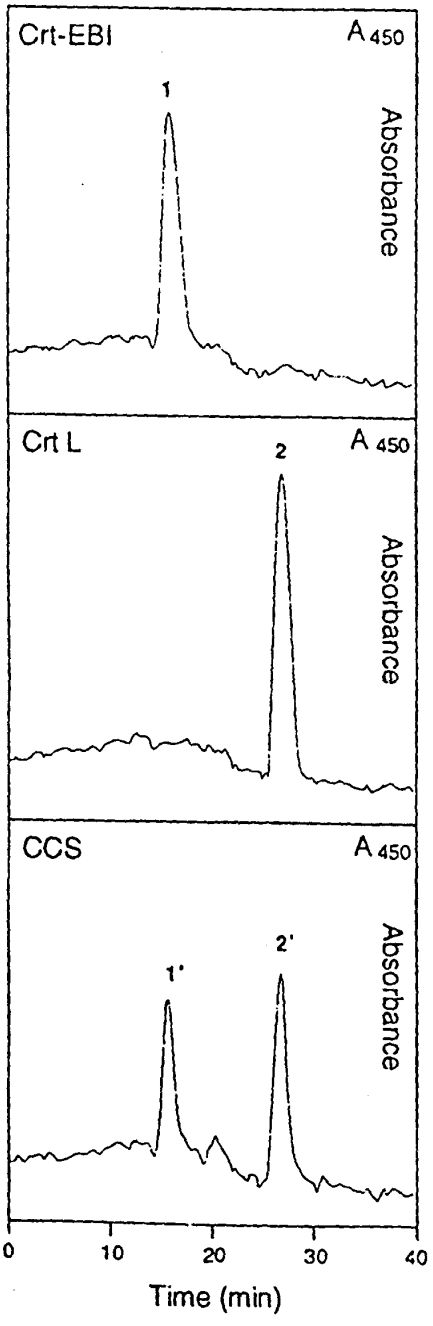


Figure 1B

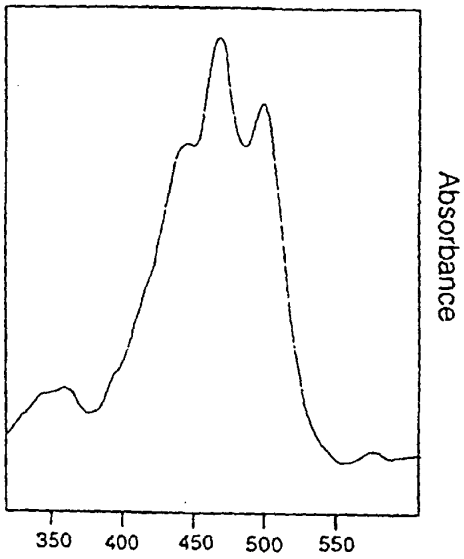
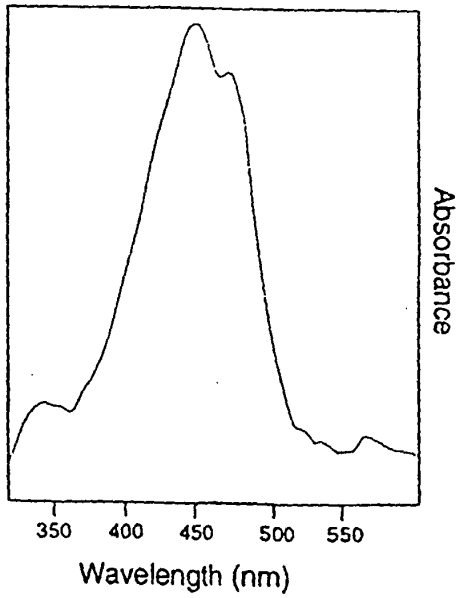


Figure 1C



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(71) Applicant *(for all designated States except US)*: CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE [FR/FR]; 3, rue Michel-Ange, F-75016 Paris (FR).

(72) Inventor; and

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(57) Abstract

The invention relates to DNA constructs comprising a DNA sequence homologous to some or all of a sequence encoding a lycopene cyclase, and to their use for modifying carotenoids levels in plants.

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INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/53 C12N15/82 C12N15/11 C12N9/02 A01H1/00

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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

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X	EMBL SEQUENCE DATABASE ACC. NO. L40176, REL.43, 15-APR-1995. ARABIDOPSIS THALIANA LYCOPENE CYCLASE (LYC) MRNA, COMPLETE CDS. XP002017204 see sequence --- -/--	3-5,9, 11-13

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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2

Date of the actual completion of the international search	Date of mailing of the international search report
4 November 1996	15. 11. 96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer Maddox, A

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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P,X	DATABASE WPI Section Ch, Week 9533 Derwent Publications Ltd., London, GB; Class C06, AN 95-250738 XP002017205 & JP,A,07 155 189 (UNIV MARYLAND BALTIMORE) , 20 June 1995 see abstract	1,3,5,9, 11-15
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	THE PLANT JOURNAL, vol. 2, no. 3, pages 343-349, XP002017201 BRAMLEY, P., ET AL.: "BIOCHEMICAL CHARACTERIZATION OF TRANSGENIC TOMATO PLANTS IN WHICH CAROTENOID SYNTHESIS HAS BEEN INHIBITED THROUGH THE EXPRESSION OF ANTISENSE RNA TO PTOM5" see the whole document ---	1-15
A	THE PLANT JOURNAL, vol. 4, no. 5, pages 833-840, XP002017202 MISAWA, N., ET AL.: "FUNCTIONAL EXPRESSION OF THE ERWINIA UREDOVORA CAROTENOID BIOSYNTHESIS GENE CRTI IN TRANSGENIC PLANTS SHOWING AN INCREASE OF BETA-CAROTENE BIOSYNTHESIS ACTIVITY AND RESISTANCE TO THE BLEACHING HERBICIDE NORFLURAZON" see the whole document ---	1-15
A	THE PLANT JOURNAL, vol. 6, no. 4, pages 481-489, XP002017203 MISAWA, N., ET AL.: "EXPRESSION OF AN ERWINIA PHYTOENE DESATURASE GENE NOT OLNLY CONFERS MULTIPLE RESISTANCE TO HERBICIDES INTERFERING EITH CAROTENOID BIOSYNTHESIS BUT ALSO ALTERS XANTHOPHYLL METABOLISM IN TRANSGENIC PLANTS" see the whole document ---	1-15
A	WO,A,91 09128 (ICI PLC) 27 June 1991 see the whole document ---	1-15
A	EP,A,0 393 690 (KIRIN BREWERY) 24 October 1990 see page 3, line 34 - line 46 see page 9, line 41 - line 42 see page 9, line 55 - page 10, line 8; claim 7 --- -/--	1-15

[redacted] Application No
PCT/EP 96/02101

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO,A,96 28014 (YISSUM RES DEV CO ;HIRSCHBERG JOSEPH (IL); CUNNINGHAM FRANCIS XAVI) 19 September 1996 see seqeunce ID nos. 4 and 5 see claims 1-71 -----	1-5,8,9, 11-15

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[redacted]ation on patent family members

[redacted]al Application No
[redacted]T/EP 96/02101

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		ES-T-	2073475	16-08-95
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